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(54) Title: ISOLATION OF AN M_r 52,000 FK506 BINDING PROTEIN AND MOLECULAR CLONING OF A CORRESPONDING HUMAN cDNA

(57) Abstract

An FK506 binding protein of mammalian origin of approximate size (M_r) 52,000, isolated by FK506 affinity chromatography and a corresponding human cDNA of approximate size 2.2 Kb, isolated by screening a human placenta cDNA library with a DNA probe whose sequence predicts a consensus amino acid sequence present in five FKBP12 sequences and in the human FKBP13 sequence.

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ISOLATION OF AN M_r 52,000 FK506 BINDING PROTEIN AND MOLECULAR CLONING OF A CORRESPONDING HUMAN cDNA

Description

Background of the Invention

FK506 and rapamycin are structurally related macrolides that block distinct steps in intracellular signalling pathways. (Sawada, S. et al., J. Immunol., 139:1797-1803 (1987); Tocci, M. J., et al., J. Immunol., 143:618-726/(1989)). Both are potent immunosuppressants, and drug action is mediated in part by binding to members of the immunophilin protein family. (Schreiber, S.L., Science, 251:283-287 (1991); Rosen, M. K. and Schrieber, S.L., Angew. Chem. Int. Ed. Engl., 31:384-400 (1992)). One recently identified FK506 binding protein (FKBP) is FKBP12 with approximate relative molecular mass (M_r) of 11,800 (12K), and a PI of 8.8-8.9. (Harding, M. W., et al., Nature, 341:758-760 (1989)). Studies have shown that the unbound FKBP12 catalyzes the cis-trans isomerization of proline residues in proteins and peptides. However, when FKPB12 binds FK506, this activity is inhibited. Recent studies suggest that the FK506-FKBP12 complex functions as an immunosuppressant by binding to, and altering, the phosphatase activity of calcineurin/calmodulin.

Summary of the Invention

The present invention relates to the isolation of an FK506 binding protein (FKBP) of mammalian origin of approximate size (M_r) 52,000 and to the molecular cloning of a corresponding human cDNA from a human placental cDNA library.

The M_r 52,000 protein, hereinafter referred to as FKBP52, is a cytosolic protein isolated from bovine thymus by FK506 affinity chromatography and is a new member of a class of immunosuppressant FK506 binding proteins that play a key role in regulating immune responses. Partial amino acid sequence of FKBP52 (approximately 30% of the complete protein sequence) is presented herein. The remaining sequence can be subsequently determined using known methods, such as those used to determine the partial sequence.

The human cDNA clone which is the subject of the present invention was isolated by screening a human placental cDNA library with a DNA probe whose sequence predicted a consensus amino acid sequence present in five FKBP12 sequences (human, murine, bovine, _Saccharomyces cerevisiae_ and _Neurospora crassa_), and in the human FKBP13 sequence, another recently identified FK506 binding protein. A clone identified in this manner contained a cDNA insert of approximately 2.2 kilobases.

The cDNA insert was purified and sequenced in its entirety. The nucleotide sequence of the coding strand (2167 bases), including the ATG initiation codon and the TAG stop codon for the deduced protein product (the correct open reading frame), is presented herein. The amino acid sequence of the protein product of the open reading frame of the human cDNA clone was deduced. The deduced protein has 459 amino acids and an M_r of 51,810, which is essentially the same M_r as that of FKBP52.

Thus, the present invention includes a M_r 52,000 FK506 binding protein (FKBP52) of mammalian origin, particularly a bovine and human M_r 52,000 protein, DNA or RNA encoding FKBP52, and nucleic acid probes which hybridize with DNA or RNA encoding FKBP52.

The present invention also includes FKBP52 homologues or equivalents (i.e., proteins which have amino acid sequences substantially similar, but not identical, to that of FKBP52 and exhibit FK506 binding characteristics). This invention further includes peptides (FKBP52 fragments which retain FK506 binding affinity, yet are less than the entire FKBP52 amino acid sequence), monoclonal and polyclonal antibodies specific for FKBP52, and uses for the nucleic acid sequences, FKBP52, FKBP52 equivalents, and FKBP52 specific antibodies. These uses include methods of screening for new immunosuppressive compounds, methods of measuring the parent compound and/or metabolites in biological samples obtained from individuals taking immunosuppressive drugs, methods of identifying natural intracellular rapamycin-like and FK506-like substances (i.e., molecules or compounds) which function in regulation of cellular metabolism, and methods of identifying natural intracellular substrates which are potential targets for other novel immunosuppressive agents.

Furthermore, as discussed herein, FKBP52 is associated with the 90kDa heat shock protein (hsp90) in untransformed steroid receptor complexes. Therefore, FKBP52 may also be useful in mediating steroidal hormone receptor transformation.

Brief Description of the Drawing

Figure 1 is the partial amino acid sequence of the M_r 52,000 protein (FKBP52). Figure 1A is the N-terminal sequence of the bovine M_r 52,000 FKBP52 (SEQ ID NO: 1). Figure 1B is the internal sequence data determined after endoproteinase Lysine C cleavage (SEQ ID NOS: 2-11).

Figure 2 depicts the deduced sequence of hFKBP52 (SEQ ID NO: 12) and 133 chemically determined residues of bFKBP52 (SEQ ID NOS: 2-11) and shows that they align well with other known FKBPs (SEQ ID NOS: 12-21), polypeptides encoded by the GenBank murine cDNAs X17068 (SEQ ID NO: 22) and X17069 (SEQ ID NO: 23), and p59 (SEQ ID NO: 24) a defined component of untransformed steroid receptor complexes. hFKBP52 shares 51 residues (above alignment) with hFKBP12, conserving 12 (dots) of the 14 residues involved in hydrogen-binding or hydrophobic interactions between hFKBP12 and FK506 or rapamycin. Nine of these residues (all except Arg42, Phe46, and Glu54) are conserved in all 15 sequences aligned here. Asterisk (*) denotes an ambiguous residue; hyphen (-) denotes a gap. (Sc = _S. cerevisiae_; Nc = _N. crassa_).

Figure 3 depicts the 2167 bp sequence of the hFKBP52 cDNA that contains 99 bp 5′ untranslated region (UTR), 1377 bp ORF, and a 691 bp 3′UTR (SEQ ID NO: 25). The deduced hFKBP52 sequence (below ORF) contains 459 residues and predicts a 51.8 kDa protein (SEQ ID NO: 26). Nucleotide and residue positions are on the left, with the initiating ATG as position 1. The TAG stop codon is identified by 3 asterisks (***), and the consensus polyadenylation-cleavage sequence AATAAA (38) is underlined. The hFKBP52 cDNA sequence has been assigned GenBank accession number M88279.

Detailed Description of the Invention

A cytosolic protein of mammalian origin of M_r 52,000 has been isolated by the Applicant on the basis of its affinity for FK506, and its partial amino acid sequence has been determined. A corresponding human cDNA has been cloned from a human placental cDNA library, its nucleic acid sequence has been determined

and the amino acid sequence of the encoded protein has been deduced. This M_r 52,000 protein is referred to herein as FKBP52 and is a member of a novel class of FK506 binding proteins of varying size and binding capabilities.

As described in detail in Example 1, affinity chromatography using an FK506 affinity matrix was performed to isolate FK506 binding proteins from mammalian tissue samples (specifically, a bovine thymus cytosolic preparation). SDS-PAGE analysis of the eluate revealed that several proteins including the M_r 52,000 protein were retained on the FK506 matrix and released by FK506 in solution.

It should be noted that using SDS-PAGE, this novel immunophilin migrated with an apparent M_r ~55,000. However, as described below, the full-length human cDNA clone was subsequently used to deduce the complete hFKBP52 amino acid sequence. This deduced amino acid sequence has a calculated M_r 51,810. Hence, the novel immunophilin described herein will be termed FKBP52 and referred to as having an M_r 52,000. FKBP52 is similar to other recently identified members of the FKBP family in this respect. The other FKBPs identified include FKBP12, FKBP13 (Jim, Y.L., et al., Proc. Natl. Acad. Sci. USA, 88:6677-6681 (1991)), and FKBP25 (Galat, A., et al., Biochem., 31:2427-2434 (1992)). These other FKBPs also each resolve as a larger protein than predicted by cDNA and/or protein sequence. Thus, referring to the novel immunophilin described herein as FKBP52 is consistent with prior convention for naming FKBPs according to their calculated M_rs.

As further described in Example 1, N-terminal amino acid sequencing of this M_r 52,000 protein (SEQ ID NO: 1) was performed after electrotransfer of the protein to a PVDF membrane, according to the method

described by Matsudaira (Matsudaira, P., J. Biol. Chem. 262:10035-10038 (1987)). In addition, internal sequence data (SEQ ID NOS: 2-11) obtained by digestion of nitrocellulose membrane-bound peptide with an appropriate endopeptidase, such as Lysine C, followed by isolation of the resulting peptide fragments using microbore HPLC techniques described by Matsudaira in A PRACTICAL GUIDE TO PROTEIN AND PEPTIDE PURIFICATION FOR MICROSEQUENCING, Academic Press (San Diego, CA, 1989)). In total, 133 amino acids of the sequence of the M_r 52,000 protein have been determined by chemical sequencing. This represents approximately 30% of the complete amino acid sequence.

Enzymatic properties of the M_r 52,000 protein (FKBP52) eluted from the FK506 affinity matrix were assessed using known methods. As described in detail in Example 2, the assay of Harrison and Stein (Harrison, R.K. and R.L. Stein, Biochemistry 29:3813-3816 (1990)) can be used to measure peptidyl prolyl cis-trans isomerization (PPIase) activity of FKBP52. Also as described in Example 2, the ability of FK506 to inhibit isomerase activity of FKBP52 was assessed, using standard techniques.

FKBP52 is an active catalyst of the PPIase reaction. Using the peptide substrate Suc-Ala-Leu-Pro-Phe-pNA, the specific activity of FKBP52 is approximately 10% that of recombinant human FKBP12 (rhFKBP12), measuring $3.9 \times 10^{5}\ M^{-1}s^{-1}$ for FKBP52 and $4.3 \times 10^{6} M^{-1}s^{-1}$ for FKBP12 at 15°C. Both FKBPs have similar selectivities for tetrapeptides differing at the P_1 position, with both immunophilins most efficiently catalyzing isomerization of peptides with large hydrophobic residues, such as leucine or phenylalanine, at P_1, as shown in Table 1.

Table 1. Characterization of hFKBP52 and hFKBP12 as PPIase catalysts of the isomerization of Suc-Ala-P1-Pro-Phe-pNA substrates

Substrate P_1	Specific activity at 15°C ($M^{-1}s^{-1}$) hFKBP12*	hFKBP52
Leu	4.3×10^6	3.9×10^5
Phe	2.0×10^6	7.3×10^4
Val	9.0×10^5	3.9×10^4
Ala	3.1×10^5	2.6×10^4

*data from Park, S.T., *et al.*, *J. Biol. Chem.*, 267:33126-3324 (1992).

The PPIase activity of hFKBP52 is potently inhibited by FK506 and rapamycin; both drugs are tight-binding inhibitors, with K_is of 10 nM and 8 nM, respectively (vs 0.6 nM and 0.25 nM, respectively, for hFKBP12). Importantly, the high affinity of FKBP52 for FK506 and rapamycin reasonably implies that FKBP52 could bind to these ligands at the systemic concentrations (blood levels) achieved during clinical use of these drugs, and that the well-documented spectrum of immunosuppressive effects and/or side-effects of FK506 therapy results, in part, from FKBP52-mediated actions.

To facilitate the isolation and determination of a human cDNA clone encoding the FKBP52 protein, DNA probes were designed as described in Example 3. A computer search was used to screen the GenPept library for peptide sequences matching a consensus pattern derived from five known FKBP12 sequences and the human FKBP13 sequence. Two murine peptides were identified in this manner.

Two DNA oligomers with sequences corresponding to part of the murine cDNA coding for the two peptides identified by the computer search were synthesized. Manually aligning these polypeptides, X17068 (SEQ ID NO: 22) and X17069 (SEQ ID NO: 23), with the 133 residues of bovine FKBP52 revealed a striking degree of sequence similarity, as shown in Figure 2.

These DNA oligomers were then used as polymerase chain reaction primers to amplify the DNA fragment. This fragment was then cloned into a cloning vector and its DNA sequence determined. This DNA fragment was then excised from the vector, radiolabeled with ^{32}P, and used to screen a human placental cDNA library (Stratagene, Catalog #936203).

As described in Example 4, a human cDNA clone containing an approximately 2.2 kb insert which hybridizes with a DNA fragment encoding a consensus amino acid sequence present in both FKBP-12 and FKBP-13, has been identified, purified, and sequenced in its entirety. The sequence of the coding strand, which is 2167 bases, is presented in Figure 3 (SEQ ID NO: 25). The correct open reading frame of the 2.2 kb cDNA sequence was identified (see Example 5) and the deduced amino acid sequence, from amino terminus to carboxyl terminus, is shown in Figure 3. The deduced protein has 459 amino acids and an M_r of 51,819 (SEQ ID NO: 26).

As described in detail in Example 6, the hFKBP52 open reading frame was expressed in *E. coli* and cleaved and uncleaved proteins were analyzed by gel electrophoresis to confirm the identity of hFKBP52. This recombinant protein migrated with an apparent M_r 55,000, just as native bovine FKBP52.

An alignment of the amino acid sequences, as determined for the bovine M_r 52,000 FK506 binding

protein, with the protein sequence predicted from the human cDNA clone, is shown in Figure 3. Comparison of the amino acid sequences revealed 89.5% sequence identity. Such sequence identity strongly suggests that the protein encoded by the isolated cDNA clone is an FK506 binding protein with characteristics substantially similar to those of the bovine FKBP52.

The original murine probe corresponded to base pairs (bps) 157-690 in the final hFKBP52 cDNA sequence, and was 89% identical to the human sequence, thus explaining its efficiency in selecting the hFKBP52 cDNA. The deduced hFKBP52 residues aligned well with the chemically determined bFKBP52 peptides, verifying the accuracy of the hFKBP52 ORF sequence and suggesting that bFKBP52 can be largely identical to the complete hFKBP52 sequence (Figure 2). Nine of the ten bovine peptides are 83-100% identical to their human homologs, while one, closest to the carboxyl terminus and perhaps reflecting relaxed structural and/or functional constraints, is 50% identical.

The deduced hFKBP52 sequence is 79% identical to the X17069 polypeptide (452 residues) and 63% identical to the X17068 polypeptide (560 residues), the lower percentage resulting from a 107 amino acid extension at the carboxyl terminus of the X17068 polypeptide (Figure 2). This indicates that the X17069 polypeptide is probably murine FKBP52 (mFKB52) while the X17068 polypeptide could be an mFKBP52-related protein or a nonexistent polypeptide reflecting a cDNA artifact.

Surprisingly, the hFKBP52 amino terminus is identical to the amino termini of two partially characterized proteins, p56 (Sanchez, E.R., _et al._, _Biochem._, 291:5145-5152 (1990)), now termed hsp 56 (Sanchez, E.R., _J. Biol. Chem._, 265:22067-22070 (1990); Yem, A.W., _et al._, _J. Biol. Chem._, 267:2868-2871

(1992)), and a reported 59 kDA immunophilin (Tai, P.-K.K., et al., Science, 256:1315-1318 (1992)), both known to associate with the heat shock protein hsp90 in untransformed steroid hormone receptor complexes.

In addition, the deduced hFKBP52 sequence is 91% identical to the predicted sequence (458 residues, in Figure 2) of p59 (Lebeau, A.-C., et al., J. Biol. Chem., 267:4281-4284 (1992)), a 59kDa protein that associates with hsp90 in the untransformed rabbit androgen, estradiol, glucocorticoid, and progesterone receptors. Therefore, it is reasonable to predict that these are all the same protein and that the deduced hFKBP52 and p59 sequences reflect the complete sequence of the 56-60 kDA protein found in untransformed mammalian and avian steroid hormone receptor complexes.

Steroid hormones bind to their respective steroid hormone receptors, and transform, or activate, the receptor to a DNA-binding form. (Sanchez, E.R., J. Biol. Chem., 265:22067-22070 (1990)). The untransformed, (in-active, non-DNA-binding) steroid hormone receptor typically comprises a receptor polypeptide associated with a number of heat shock proteins (hsps), with a sedimentation coefficient of approximately 9S. For example, the glucocorticoid receptor is a heterotetramer with one receptor polypeptide, two hsp 90 molecules and on hsp 59 molecule. (Rexin, M. et al., J. Biol. Chem., 266:24601-24605 (1990). Upon binding of steroid hormone to untransformed receptor, the 9S complex dissociates to a ~4-6S form, which then binds to DNA. As a component of untransformed steroid receptor complexes, FKBP52 could be involved in stabilizing, or blocking, the inactive receptor and this could affect conversion of the receptor to its active, DNA binding, state by binding FK506 and/or rapamycin.

Furthermore, the deduced hFKBP52 sequence reveals a core consensus region when aligned with FKBP12 and other FKBPs. This consensus region lies within the amino terminal portion of hFKBP52, between residues 41-134, and contains 51 residues of conserved identity and position (Figure 2). The key residues contributing to the high-affinity interaction between hFKBP12 and FK506 corroborate this FKBP12-like core of hFKBP52 and reasonably predict that residues 41-134 define the FK506- and rapamycin-binding domain of hFKBP52.

The residues critical to the hFKBP12-FK506 interaction, defined by high resolution structural analysis of the complex, and site-directed mutagenesis studies of individual hFKBP12 residues, are highly conserved in the hFKBP52 core region. Thirteen of the fourteen residues involved in hydrogen bonding or hydrophobic interactions between hFKBP12 and FK506 (Tyr26, Phe326, Asp37, Arg42, Phe46, Gln53, Glu54, Val55, Ile56, Trp59, Tyr82, His87, Ile91, and Phe99 in FKBP12) are conserved in FKBP52 (dotted residues, Figure 2). The high degree with which these crucial residues are conserved reasonably explains why rhFKBP52 displays a high affinity for FK506 and rapamycin and similar substrate specificity profile for PPIase catalysis.

A pattern search alignment algorithm and secondary structure analysis (DNAStar, Inc. software) also corroborate the hFKBP52 homology alignment. Pattern searching, built around the positions and identities of hFKBP12 residues that interact with FK506 and are conserved in different FKBP12 sequences, aligned the FKBP12, p59, and X17069 polypeptide sequences. Secondary structure analysis of the hFKBP52 sequence predicted that the first one-third of the protein contains the FKBP12-like domain. The Trp59 residue of

FKBP12, in Van der Waals contact with the pipecolinic moiety of FK506 and completely conserved in all FKBPs (Figure 2), was a particularly useful benchmark of the latter analysis. In all known members of the FKBP family, this conserved Trp residue is found near the beginning of a short α-helix that follows a short β-sheet.

The 325 residues of hFKBP52 that lie beyond the FKBP12 consensus region reasonably form at least one additional protein domain. Hydrophobic cluster analysis (HCA) has been used to postulate that p59, the rabbit homolog of hFKBP52, has three hsp binding immunophilins (HBI) domains structurally related to FKBP12. They define the first domain, HBI-I, as hFKBP52 residues 32-138 and predict that the second and third domains, HBI-II and HBI-III, correspond to residues 149-253 and 268-372, respectively. The HBI-I domain clearly corresponds well to the core consensus region of residues 41-134 that were defined for hFKBP52 by sequence alignments. Furthermore, the model predicts that the remaining residues of hFKBP52 will be organized as two domains, each with structural similarities to the first.

Given that FK506 and rapamycin bind to untransformed glucocorticoid receptor complexes without displacing the integral components, that FKBP52 associates directly with hsp90 (Renoir, J.-M., <u>et al.</u>, <u>J. Biol. Chem.</u>, 265:10740-10745 (1990), Rexin, A., <u>et al.</u>, <u>J. Biol. Chem.</u>, 266: 24601-24605 (1991)), and that FK506 and rapamycin bind directly to FKBP52, it is reasonable to predict the FKBP52 will have at least two structural domains to accommodate these distinct functions. The FKBP12-like consensus region in the first one-third of FKBP52 reasonably defines the immunosuppressant binding domain of the protein, while

the remaining residues reasonably constitute the putative hsp90 binding site.

The deduced hFKBP52 sequence contains a variety of consensus motifs that reflect possible post-translational modification(s) and/or functional characteristics of the protein. Consensus motifs typical of asparagine-linked glycoproteins, protein kinase phosphorylation sites, and calmodulin binding domains are present.

Moreover, fourteen protein kinase phosphorylation site elements, representing five classes of motifs, are present in the deduced hFKBP52 sequence. Using asterisks to identify potentially phosphorylated residues and "X" to denote any amino acid, these sites are as follows: $L^{317}RLAS^{*}H$, a multifunctional calmodulin-dependent protein kinase II or S6 kinase II element ($XRXXS^{*}X$); $I^{25}S^{*}PK$ and $G^{117}S^{*}PP$, a proline-dependent protein kinase motif ($XS^{*}PX$); $G^{114}SAGS^{*}P$, $W^{259}EMNS^{*}E$, $L^{300}EYES^{*}S$, $E^{393}SSFS^{*}N$, $L^{346}ELDS^{*}N$, $A^{427}EASS^{*}G$ and $E^{442}EQKS^{*}N$, casein kinase I phosphorylation sites ($XS(P)XXS^{*}X$ or $XEXXS^{*}X$); and $V^{297}S^{*}WLEY$, $F^{306}S^{*}NEEH$, $D^{349}S^{*}NNEK$, and $Q^{452}S^{*}QVET$, sites of casein kinase II (CKII) phosphorylation ($XS^{*}XXEX$). These motifs suggest that the ~59 kDa immunophilin is phosphorylated and that phosphorylation(s) could produce multiple isoforms. Since hsp90 associates with, and enhances CKII kinase activity of, CKII in cell lysates and in *in vivo* reconstitution assays, it is reasonable to predict that CKII associates *in vivo* with an hsp90-FKBP52 complex and phosphorylates one or more serines in both proteins. The putative calmodulin binding domain of p59 (Lebeau, M. C., *et al.*, *J. Biol. Chem.* 267:4281-4284 (1992)), suggests that the seventeen residue stretch Arg399-Phe415 comprises a similar domain in hFKBP52. These residues constitute an amphilili*c* α-

helical peptide, a motif common to many calmodulin-binding proteins (O'Neil, K.T., et al., Trends. Biochem-Sci., 15:59-64 (1990)), and suggest that calmodulin and intracellular Ca^{+2} levels could modulate hFKBP52 function.

Thus, as described above, a new member of the class of FK506 binding proteins has been identified and shown to be of approximate M_r 52,000. A human cDNA clone containing a cDNA insert which hybridizes with a DNA fragment encoding a consensus amino acid sequence present in both FKBP12 and FKBP13 has also been obtained and its deduced amino acid sequence has been shown to encode a protein of size M_r 51,810, essentially the same as that of the binding protein isolated by FK506 affinity chromatography (M_r 52,000).

This human cDNA clone can be used to produce an FKBP52 in vitro, such as by introducing the insert into an appropriate expression vector (e.g., pKK223, pOP, pRK5B) and expressing the encoded product in host cells (bacterial, yeast, or mammalian) containing the expression vector. This expressed FKBP52 can be used for a number of diagnostic and therapeutic purposes.

The FKBP52 can be used in screening assays for detection of new naturally occurring immunosuppressant compounds. For example, FKBP52 could be used to screen fermentation broths, produced by known techniques, for compounds that bind to it and, thus, are potential immunosuppressant candidates. Alternately, FKBP52 can be used to screen existing synthetic compounds for binding affinity and subsequent immunosuppressant evaluation. It is reasonable to expect that a compound which binds FKBP52 will be FK506-like and, thus, have immunosuppressive capabilities.

FKBP52 can also be used as the basis for design of FK506-like molecules by determining and characterizing

the active binding site(s) of FKBP52, designing a molecule which binds to it (them) and assessing its ability to suppress an immune response.

It is also possible to use the newly identified FKBP52 for diagnostic purposes. For example, FKBP52 can be affixed to a solid support using a variety of chemical coupling techniques which link amino acid residues, such as methionine, lysine, cystine, and tryptophan to inert matrixes, such as Affigel (BioRad) or cyanogen bromide-treated Sepharose (Pharmacia). The FKBP52 bearing solid support is then contacted with tissue extracts or body fluids, such as blood and urine, from individuals receiving FK506 immunosuppressant treatment. Detection and/or quantitation of the parent compound FK506, or its metabolites, can be carried out using known methods, such as spectrophotometric measurement or scintillation counting.

It is also possible to use FKBP52 to identify natural, intracellular FK506-like substances (i.e., molecules or compounds) that function in intrinsic regulatory events in cellular immunity and metabolism. FK506-like substances are defined herein as substances which bind FKBP52 to a similar extent as FK506 under the same conditions under which FK506 binds with FKBP52. Furthermore, FKBP52 can be used to identify natural intracellular substances that may be targets for other novel immunosuppressive agents.

FKBP52 can also be modified in such a way as to enhance its binding capability, and/or other immunosuppressive characteristics. Such modifications (e.g., truncating sequence length) can be carried out using known methods, such as site directed mutagenesis.

Finally, FKBP52 can be used to modify the transformation of steroid hormone receptors. As

discussed herein, FKBP52 is common to several vertebrate species and is associated with the 90kDa heat shock protein (hsp90) in untransformed steroid hormone receptors. Thus, it is reasonable to predict that FKBP52 plays a critical role in the transformation of steroid hormone receptors.

For example, evidence presented herein indicates that FK506 binds tightly to FKBP52. (FKBP52 also binds rapamycin, another immunosuppressive agent). It is also established that certain immunosuppressive treatments (e.g., cyclosporin, which binds to the immunophilin, cyclophilin) result in unpleasant side-effects which can be attributed to an increase in steroid hormone levels. (Paus, R., et al., Lab. Invest., 60:365-369 (1989)). It is reasonable to predict that FK506 binds to FKBP52, which, in turn, transforms a steroid hormone receptor by causing dissociation of the FKBP52 molecule from a steroid hormone receptor complex, such as the androgen receptor. This transformation of the steroid receptor could lead to unwanted side-effects, such as an increase in body hair growth. An antibody to FKBP52 can be co-administered to an individual receiving FK506 therapy to block binding of FK506 to FKBP52 and consequently block the steroid hormone receptor transformation. Alternatively, an FK506-like substance, can be used as an antagonist to block FK506 binding to FKBP52.

It is also possible to design an anti-sense nucleotide which will hybridize to the mRNA encoding FKBP52, and inhibit translation of the mRNA to protein. Thus, production of FKBP52 can be decreased, or completely inhibited, thereby decreasing, or eliminating unwanted steroidal side-effects during FK506 or rapamycin therapy.

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLE 1 Protein Purification and Sequencing

An amino derivative of FK506 at the C32 position was prepared as described in Fretz et al. (Fretz, H. et al., J. Am. Chem. Soc. 113:1409-1411 (1991)) and coupled to Affigel 10 resin to yield an FK506 affinity matrix (approximately 1 mg of FK506 coupled per ml of resin). Bovine thymus cytosol extract was prepared as follows: tissues were snap-frozen in liquid nitrogen, and 75 gram amounts were homogenized in 100 mM potassium phosphate, pH 7.4, containing 1 mM PMSF and 5 mM DTT for 60 sec in a Waring blender. The extract was clarified by centrifugations at 40,000 x g and then 100,000 x g. Cytosol extract was then passed over a 5 ml FK506 affinity column containing an amino acid derivative of FK506 at the C32 position. Flow rate was 0.2 ml/min. The column was washed extensively with phosphate buffered saline containing 0.1% Tween 20 detergent and eluted sequentially with FK506 (200 μg/ml in phosphate buffer) and then 6 M guanidine hydrochloride. Eluted proteins were dialyzed extensively against 10 mM Tris, pH 7.0, and aliquots were lyophilized. Approximate molecular weight was determined by SDS-PAGE on a 12½% acrylamide gel using lysozyme (M.W. 14,400), α-chymotrypsin (M.W. 21,500), carbonic anhydrase (M.W. 31,000), ovalbumin (M.U. 45,000) and bovine serum albumin (M.W. 66,000) to calibrate relative migration.

Proteins were visualized by Coomassie blue or silver staining or electroblotted onto either Immobilon-P (0.45 μm pore size, Millipore) or nitrocellulose (Schleicher and Scheull). The proteins

transferred to Immobilin-P were visualized by Coomassie blue and used for N-terminal sequencing, described below. Proteins transferred to nitrocellulose were visualized with Ponceau S and used for in situ digestion.

N-terminal amino acid sequencing was performed after electrotransfer to a PVDF membrane as described by Matsudaira, P., J. Biol. Chem., 262:10035 (1987). A band of protein with M_r - 55,000 band was excised from the Immobilin -P membrane and loaded directly into an automated sequencer (Applied Biosystems) for amino terminal sequencing. For internal sequence determination, peptide fragments were generated by digest M_r-55,000 band (on nitrocellulose) with endoproteinase Lysine C (Wako Chemicals, USA) and then separating them by an HPLC system (Hewlett Packard) equipped with a variable wavelength detector and a Vydac C18 2.1 x 250 mm column. A two-step linear gradient was used to elute the peptides; buffer A was 0.09% trifluoroacetic acid (TFA) in water while buffer B was 0.06% TFA in acetonitrile. Peptides were eluted at a flow rate of 200 μl min^{-1} with a sequence of linear gradients from 5% B at 0 min to 33% B at 65 min, 60% B at 90 min, and 100% B at 105 min. Peaks absorbing at 214 nm were collected in 0.5 ml microcentrifuge tubes and stored immediately without drying at -20°C. For protein sequence determination, the peak fractions were applied to a polybrene precycled glass-fiber filter and placed in the sequencer reaction cartridge. The N-terminal amino acid sequence (SEQ ID NO: 1) and additional internal sequences (SEQ ID NOS: 2-11) of the Mr 52,000 protein are shown in Figure 1.

EXAMPLE 2 Peptidyl-prolyl cis-trans Isomerase Assay

The peptidyl-prolyl isomerization rate was determined by coupling isomerization of a prolyl-containing peptide to trans substrate hydrolysis by chymotrypsin (Fisher, G., et al., Nature 337:476-478 (1989)). The assay was performed according to Harrison and Stein (Biochem., 29:3813-3816 (1980) with modifications described by Park S. T., et al., J. Biol. Chem. 267:3316-3324 (1992). The tetrapeptide substrate succinyl-Ala-P1-Pro-Phe-p-nitroanilide, where P1 = Leu, was used to determine specific activity and inhibition constants, and a series of peptide substrates with related structures (P1 = Phe, Val, Ala, Gly, Glu or Lys) was used to determine substrate specificity. Protein concentrations of rhFKBP52 stock solutions were determined by a Coomassie Blue binding assay (Bradford, M., Anal. Biochem., 72:248-254 (1976)). FKBP52 (60 nM final) was added to a reaction mixture containing substrate (27 μM final) in 0.1 M Tris-HCl, pH 7.8 at 15°C, and the solution was incubated in a 2 ml cuvette for 5 min at 15°C (950 μl final) before adding chymotrypsin (100 μg ml^{-1} final) to start reaction. For measurement of substrate specificity, the final FKBP52 concentration was adjusted so the K_{obs} was at least four-fold higher than $k_{non\text{-}enz}$. Inhibition data were fit to an equation for tight-binding competitive inhibitors using KineTic™ software (BioKin, Ltd.) running on a Macintosh IIcx computer.

EXAMPLE 3 Identification of Murine cDNA Sequences

A computer search was undertaken to identify protein sequences that contain a consensus pattern of conserved residues derived from five FKBP12 sequences (human, murine, bovine, Saccharomyces cerevisiae and

Neurospora crassa) and the human FKBP13 sequence. This consensus pattern (SEQ ID NO: 32) is as follows:

lG-xxx-xxxx-xxxx-xxxGxxxxxHYxGxLxxGxxFDxSxxxxxPxxxxxGx-Q-

VIXGWxxGxxxxxxGxxxxLxIx-x-xxYGxxxxxxxIPxxxTLxFxxELx------Kxx

The residues indicated in upper case letters are specific amino acids, defined by the single letter amino acid code. Each dash (-) indicates a gap introduced into one or more of the protein sequences for optimal alignment. A cross mark (x) represents any amino acid. Of the thirty-one conserved amino acids defined by the consensus pattern, nine Y26, F36, D37, V55, I56, W59, Y82, I91 and F99 (the upper case letter is the amino acid and the number is the position of the residue within human FKBP12) are residues known to interact with FK506 in the human FKBP12/FK506 co-complex (Van Duyne, G.D. et al., Science 251:839 (1991)). When a computer search was performed on the translated GenBank database (GenPept) using the above consensus pattern for alignment, the predicted protein products of two murine cDNA sequences (GenBank accession number X17068 and X17069) were identified. These predicted protein products are identical to each other because the first 1300 base pairs (bp) of X17068 and X17069 are identical. X17068 is 1817 bp in length (SEQ ID NO: 22); X17069 is 2046 bp in length (SEQ ID NO: 23). The alignment of the consensus sequence with the homologous portions of the predicted protein products from X17068 and X17069 is shown below:

Consensus

G-xxx-xxxx-xxxx-xxxGxxxxxHYxGxLxxGxxFDxSxxxxxPxxxxxGx-Q-

X17068

G-VLKVIKREGTGTETPMIGDRVFVHYTGWLLDGTKFDSSLDRKDKFSFDLGK-GE

X17069

G-VLKVIKREGTGTETPMIGDRVFVHYTGWLLDGTKFDSSLDRKDKFSFDLGK-GE

Consensus

VIxGWxxGxxxxxxGxxxxLxIx-x-xxYGxxxxxxxIPxxxTLxFxxELx------Kxx

X17068

VIKAWDIAVATMKVGEVCHITCK-PEYAYGAAGSPPKIPPNATLVFEVELFFEF---KGE

X17069

IKAWDIAVATMKVGEVCHITCK-PEYAYGAAGSPPKIPPNATLVFEVELFFEF---KGE

The predicted protein products from X17068 and X17069 were also identified by searching the GenPept database directly within the human FKBP12 amino acid sequence (SEQ ID NO: 13). The alignment of the human FKBP12 (hFKBP12) sequence with the homologous portions of the predicted protein products of X17068 and X17069 is shown below:

hFKBP12
GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQE

X17068
GVLKVIKREGTGTETPMIGDRVFVHYTGWLLDGTKFDSSLDRKDKFSFDLGKGE

X17069
GVLKVIKREGTGTETPMIGDRVFVHYTGWLLDGTKFDSSLDRKDKFSFDLGKGE

hFKBP12
VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE

X17068
VIKAWDIAVATMKVGEVCHITCKPEYAYGAAGSPPKIPPNATLVFEVELFFEF

X17069
VIKAWDIAVATMKVGEVCHITCKPEYAYGAAGSPPKIPPNATLVFEVELFFEF

Of the fifty-four conserved amino acids defined by this alignment, fifteen (Y26, F36, D37, R42, F46, F48, Q53, E54, V55, I56, W59, Y82, H87, I91 and F99) are residues known to interact with FK506 in the human FKBP12-FK506 co-complex (Van Duyne, G.D. et al., 1991)).

EXAMPLE 4 Isolation of Human cDNA Encoding FKBP52

Two short DNA oligomers, each selected from a 1300 bp region of identity within X17068 and X17069 cDNAs, were synthesized as PCR primers. The oligomers SEQ ID NO: 27, forward primer, and SEQ ID NO: 28, reverse primer) were constructed on a DNA synthesizer (Applied Biosystems) and used to amplify an approximate 500 bp fragment from a λZAPII mouse thymus cDNA library (Stratagene Cloning Systems). To amplify the DNA, 2μl of the library was heated at 80°C in 33.7 μl of water for 15 min. and the primers (0.4 μM final), reaction buffer, dNTPs, and AmpliTaq were added according to Gene-Amp PCR reagent kit (Perkin-Elmer Corporation) instructions. The DNA was amplified in a thermocycler (Eppendorf) for 35 rounds (cycles of 94°C for 1 min, 58°C for 2 min, 72°C for 2 min), and the resultant fragment was resolved on a 3% agarose gel (NuSieve 3:1, FMC Bioproducts), transferred to GeneScreen (DuPont-New England Nuclear), and hybridized with a radiolabeled oligomer SEQ ID NO: 29 that was predicted from the X17068 and X17069 sequences to be internal to the fragment. When autoradiography demonstrated specific hybridization, the fragment was cloned into pCR1000 (Invitrogen Corporation), and competent E. coli DH5α was transformed and plated. The cloned insert (positive colony identified by hybridization), corresponding exactly to a 534 bp portion of the murine cDNAs (nucleotides 40-573 in X17068 and X17069), was sequenced with a Sequenase Version 2.0 DNA sequencing kit (US Biochemicals).

The fragment was excised with EcoR I and Hind III (all restriction enzymes from New England BioLabs), radiolabeled with ^{32}P dCTP, and used as a hybridization probe for library screening. Eighteen clones were selected by screening 4 x 10^5 plaques of a human

placenta λZAPII cDNA library (Stratagene) under stringent conditions. Fifteen clones were rescreened, and the inserts of twelve were excised to produce pBluescript (Stratagene) subclones for sequence analysis. Purified DNA from each clone was digested with Sac I and Kpn I, and insert sizes were determined by agarose gel electrophoresis. Partial nucleotide sequences of each insert were determined with universal sequencing primers and the Sequenase kit. A human FKBP52 (hRKBP52) cDNA clone containing an approximate 2.2 kilobase (kb) insert with 73% identity to the X17068 and X17069 nucleotide sequences was purified and sequenced in its entirety. The sequence of the coding strand of the human cDNA clone, from 5′ to 3′, is shown in Figure 3 (SEQ ID NO: 25).

The sequence is 2167 bases in length. The ATG initiation codon and the TAG stop codon for the deduced protein product are underlined.

<u>EXAMPLE 5</u> <u>Deduced Amino Acid Sequence from the Human cDNA Clone</u>

The correct open reading frame of the human cDNA sequence was identified by comparing the possible translation products to (1) the determined peptide sequences from the bovine thymus M_r 52,000 protein and 2) the deduced amino acid sequences of the murine cDNAs identified by computer search. The deduced amino acid sequence, from amino terminus to carboxyl terminus, of the human protein is shown in Figure 3 (SEQ ID NO: 26).

EXAMPLE 6 Expression and Purification of Human FKB52 From E. Coli

The hFKBP52 open reading frame (ORF) was expressed in E. coli with a vector (pQE8, Qiagen Inc.) that expresses recombinant proteins with an amino terminal histidine tag that facilitates protein purification via Ni^{2+} affinity chromatography. By modifying the 5′ end of the hFKBP52 ORF to encode a cleavage site, we could use Factor Xa to remove the tag and cleavage site from the recombinant hFKBP52 (rhFKBP52). Synthetic oligomers were used as PCR primers to modify and amplify the ORF. The forward primer, SEQ ID NO: 30, included a BamHI site (GGATCC), nucleotides encoding the Factor Xa cleavage site (ATCGAGGGTAGA to encode Ile-Glu-Gly-Arg), and the first nineteen nucleotides of the hFKBP52 ORF (ATGACAGCCGAGGAGATGA). The reverse primer, SEQ ID NO: 31, included a Hind III site (AAGCTT) and the complement of a stop condon (TTA) followed by the complement of the last sixteen nucleotides of the hFKBP52 ORF (TGCTTCTGTCTCCACC).

The ORF was amplified from the hFKBP52 insert by 10 rounds of PCR (5 min denaturation at 94°C for 1 min, 72°C for 2 min, final extension at 72°C for 10 min) in a thermocycler (Perkin Elmer Corporation), and the resultant DNA fragment was digested with BamH I and Hind III, cloned into the BamH I and Hind III sites of pQE8, and used to transform E. coli XA90 (the kind gift of J. Wang, Harvard University). A 500 ml volume of Luria broth (100 μg ml^{-1} amplicillin) was inoculated with a positive colony, and the culture was grown at 37°C to OD_{600} 0.6. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 2mM, and the cells were grown for an additional 2 hr before harvesting by centrifugation (4,000 x g, 20 min, 4°C). The cells were lysed by stirring for 1 hr at room temperature in

6 M guanidine HCl, 0.1 M NaH_2PO_4, 10 mM Tris adjusted to pH 8.0 with NaOH, and the lysate was cleared by centrifugation (10,000 x g, 15 min, 4°C) and applied to an 8 ml Ni^{2+}-NTA-agarose (Qiagen Inc.) affinity column. rhFKBP52 was eluted from the column according to the manufacturer's instructions and was refolded by dialysis against Factor Xa buffer (0.1M NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$) for 3 hr at 4°C. The amino terminal tag was removed by dissolving ~30μg of lyophilized Factor Xa (Boehringer·Mannheim Biochemicals) in 5 ml of the refolded protein and then dialyzing twice overnight at 4°C against Factor Xa buffer. Cleaved and uncleaved protein was analyzed by gel electrophoresis and amino terminal sequencing to confirm the identity of the rhFKB52.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. Homogeneous protein of mammalian origin of approximate M_r 52,000 which binds FK506.

2. The homogeneous protein of Claim 1 in which the N-terminal 27 amino acids are SEQ ID NO: 1.

3. The homogeneous protein of Claim 2 which includes the following internal amino acid sequences:

a) SEQ ID NO: 2
b) SEQ ID NO: 3
c) SEQ ID NO: 4
d) SEQ ID NO: 5
e) SEQ ID NO: 6
f) SEQ ID NO: 7
g) SEQ ID NO: 8
h) SEQ ID NO: 9
i) SEQ ID NO: 10
j) SEQ ID NO: 11

where xxx designates an indeterminate amino acid.

4. The homogeneous protein of Claim 1, which is of human origin.

5. Recombinantly produced protein of mammalian origin of approximate M_r 52,000 which binds FK506.

6. The recombinantly produced protein of Claim 5 in which the N-terminal 27 amino acids are SEQ ID NO: 1.

7. The homogeneous protein of Claim 6 which includes the following internal amino acid sequences:
a) SEQ ID NO: 2
b) SEQ ID NO: 3
c) SEQ ID NO: 4
d) SEQ ID NO: 5
e) SEQ ID NO: 6
f) SEQ ID NO: 7
g) SEQ ID NO: 8
h) SEQ ID NO: 9
i) SEQ ID NO: 10
j) SEQ ID NO: 11

8. The recombinantly produced protein of Claim 5, which is of human origin.

9. cDNA of mammalian origin which encodes a M_r 52,000 protein which binds FK506.

10. A cDNA clone of Claim 9 which is of human origin.

11. A cDNA clone of Claim 10 containing a cDNA insert which hybridizes to the nucleotide sequence SEQ ID NO: 25.

12. A cDNA clone of mammalian origin containing a cDNA insert which hybridizes to DNA encoding an amino acid sequence present in FKBP12.

13. A cDNA clone of Claim 12 in which the cDNA insert hybridizes to DNA encoding the amino acid sequence SEQ ID NO: 26).

14. The cDNA clone of Claim 12 which is of human origin.

15. Isolated DNA of mammalian origin encoding a M_r 52,000 protein which binds FK506.

16. An isolated DNA sequence of Claim 15 of bovine origin.

17. An isolated DNA sequence of Claim 15 of human origin.

18. An isolated DNA sequence of Claim 15 comprising the nucleotide sequence SEQ ID NO: 25.

19. A method of identifying an FK506-like substance, comprising the steps of:

a) combining a substance to be tested with FKBP52 which is bound to a solid support;

b) maintaining the product of (a) under conditions appropriate for binding of FKBP52 with FK506; and

c) determining whether binding of FKBP52 and the substance being tested occurred in step (b), wherein binding is indicative of the presence of an FK506-like substance.

20. The method of Claim 19 wherein FKBP52 is bound to the solid support by a chemical linkage to an FKBP52 amino acid residue.

21. A method of detecting FK506 in a biological sample, comprising the steps of:
    a) combining the biological sample with FKBP52 which is bound to a solid support;
    b) maintaining the product of (a) under conditions appropriate for binding of FKBP52 with FK506; and
    c) determining whether binding of FKBP52 and FK506 occurred in step (b), wherein binding is indicative of the presence of FK506.

22. DNA whose sequence encodes a consensus amino acid sequence (SEQ ID NO: 32) present in human FKBP12 (M_r 12,000) and in human FKBP13 (M_r 13,000).

23. DNA encoding a FKBP which hybridizes to a consensus amino acid sequence (SEQ ID NO: 32) present in human FKBP12 (M_r 12,000) and in human FKBP13 (M_r 13,000).

24. Use of an anti-FKBP52 antibody for the manufacture of a medicament for inhibiting steroid hormone receptor transformation in FK506 therapy.

25. Use of an FK506-like antagonist substance for the manufacture of a medicament for inhibiting steroid hormone receptor transformation in FK506 therapy.

26. A method of inhibiting steroid hormone receptor transformation, comprising blocking binding of an FKBP with FK506, thereby blocking transformation of the hormone receptor.

27. The method of Claim 26 wherein FKBP52 binding with FK506 is blocked.

28. The method of Claim 27 wherein an anti-FKBP52 antibody binds to FKBP52, thereby blocking binding of FKBP52 with FK506.

29. The method of Claim 27 wherein FKBP52 binding with FK506 is blocked with an FK506-like substance which does not transform the steroid hormone receptor.

FIGURE 1

Figure 1A

N-terminal sequence

H_2-N-Thr-Ala-Glu-Glu-Thr-Lys-Ala-Ala-Glu-Ser-Gly-Ala-Gln-Ser-Ala-Pro-Leu-Arg-Leu-Glu-Gly-Val-Asp-Ile-Ser-Pro-Lys_{27}

Figure 1B

1. Asp-Arg-Phe-Ser-Phe-Asp-Leu-Gly-Lys
2. Ala-xxx-Asp-Ile-Ala-Val-Ala-Thr
3. Met-Lys-Val-Gly-Glu-Val-xxx-His-Ile-Thr-Cys-Lys
4. Ile-Pro-Pro-Asn-Ala-Thr-Leu-Val-Phe-Glu-Val-Glu-Leu-Phe-Glu-Phe-Lys
5. Pro-Asn-Glu-Gly-Ala-Leu-Val-Glu-Val-Ala-Leu-Glu-xxx-Tyr-Phe-Gln
6. Tyr-Glu-Ile-His-Leu-Lys
7. Gly-Thr-Val-Tyr-Phe-Lys
8. Ala-Leu-Glu-Leu-Asp-Ser-Asn-Asn-Glu-Lys
9. Leu-Tyr-Ala-Asn-Met-Phe-Glu-Leu-Ala-Ala-Glu-Glu-Glu-xxx-Lys
10. Ala-Leu-Val-Ala-Ala-Gly-Asp-Gln-Pro-Ala-Asp-Ala-Glu-Met-Arg-Asp-Glu-Pro

Internal sequence of peptides

(xxx=indeterminate amino acid)

Figure 2

```
                                                                                                  o                     oo                    o         o              ooo   o
Consensus                                                                      G  G    P  G     VHYTG  L   DG KFD       SS  DR         F F L GK EVI    W
hFKBP52   M TAEEMKATES GAQSAPLPHE GVDISPKQDE GVL--KVIKREG TGTEMPMIGD RVFVHYTGW-L LDGTKFD-----SSL DR-K-DKFSFDL GKGEVIKAWD9.
bFKBP52     TAEETKAAES GAQSAPLRLE GVDISPK                                                          DRFSFDL GK      A*D
hFKBP12                                      GVQ--VETISPG DGRTFPKRGQ TCVVHYTGM-L EDGKKFD-----SSR DR-N-KPFKFML GKQEVIRGWE60
mFKBP12                                    M GVQ--VETISPG DGRTFPKRGQ TCVVHYTGM-L EDGKKFD-----SSR DR-N-KPFKFTL GKQEVIRGWE
bFKBP12                                      GVQ--VETISPG DGRTFPKRGQ TCVVHYTGM-L EDGKKFD-----SSR DR-N-KPFKFVL GKQEVIRGWE
ScFKBP12                        M SEVIEGNVK- ------IDRISPG DGATFPKTGD LVTIHYTGT-L ENGQKFD-----SSV DR-G-SPFQCNI GVGQVIKGWD
NcFKBP12                        M TIPQLD---- GLQ--IEVQQEG QGTRETRRGD NVDVHYKGV-L TSGKKFD-----ASY DR-G-EPLNFTV GQGQVIKGWD
hFKBP13     MRLSWFRVLT VLSICLSAVA TATGAEGKRK LQIGVKKR--VD HCPIKSRKGD VLHMHYTGK-L EDGTEFD-----SSL PQ-N-QPFVFSL GTGQVIKGWD
bFKBP13                             TGTEGKRK LQIGVKKR--VD HCPIKSRKGD VLHMHYTGK-L EDGTEFD-----SSL PQ-N-QPFVFSL GTGQVIK
ScFKBP13    MHFNIYLFVT FFSTIL---- --- AGSLSD LEIGIIKRIPVE DCLIKAMPGD KVKVHYTGSLL ESGTVFD-----SSY SR-G-SPIAFEL GVGRVIKGWD
hFKBP25     KVSEQVKNVK LNEDKPKETK SEETLDEGPP KYT--KSVLKKG DKTNFPKKGD VVHCWYTGT-L QDGTVFDTNIQTSAK KKKNAKPLSFKV GVGKVIRGWD
X17068           ARGGG ERGAVGVPLE GVDISPKQDE GVL--KVIKREG TGTETPMIGD RVFVHYTGW-L LDGTKFD-----SSL DR-K-DKFSFDL GKGEVIKAWD
X17069           ARGGG ERGAVGVPLE GVDISPKQDE GVL--KVIKREG TGTETPMIGD RVFVHYTGW-L LDGTKFD-----SSL DR-K-DKFSFDL GKGEVIKAWD
p59       M TAEEMKAAES GAQSAPLPLE GVDISPKQDE GVL--KVIKREG TGTETPMIGD RVFVHYTGW-L LDGTKFD-----SSL DR-K-DKFSFDL GKGEVIKAWD

                                        o             o        o
Consensus     A M VG       T  P Y AYG  G P      IPP ATLVF  VEL
hFKBP52   IAIATMKVGE VCHITCKPEY AYGSAGSPP-K IPPNATLVFE VELFEFKGED LTEEEDGGII RRIQTRGEGY AKPNEGAIVE VALEGYYKDK LFDQRELRFE191
bFKBP52    IAVATMK                          IPPNATLVFE VELFEF                                  PNEGALVE VALE
hFKBP12    EGVAQMSVGQ RAKLTISPDY AYGATGHPG-I IPPHATLVFD VELLKLE107
mFKBP12    EGVAQMSVGQ RAKLIISSDY AYGATGHPG-I IPPHATLVFD VELLKLE
bFKBP12    EGVAQMSVGQ RAKLTISPDY AYGATGHPG-I IPPNATLIFD VELLKLE
ScFKBP12   VGIPKLSVGE KARLTIPGPY AYGPRGFPG-L IPPNSTLVFD VELLKVN
NcFKBP12   EGLLGMKIGE KRKLTIAPHL AYGNRAVGG-I IPANSTLIFE TELVGIKGVQ KGE
hFKBP13    QGLLGMCEGE KRKLVIPSEL GYGERGAPP-K IPGGATLVFE VELLKIERRT EL
bFKBP13           EGE KRKLVIPSEL GYGERGAPP-K
ScFKBP13   QGVAGMCVGE KRKLQIPSSL AYGERGVPG-V IPPSADLVFD VELVDVKSAA
hFKBP25    EALLTMSKGE KARLEIEPEW AYGKKGQPDAK IPPNAKLTFE VELVDID
X17068    IAVATMKVGE VCHITCKPEY AYGAAGSPP-K IPPNATLVFE VELFEFKGED LTEEEDGGII RRIRLGVKAM QGPNDGAMVE VALEGYHKDR LFDQRELCFE
X17069    IAVATMKVGE VCHITCKPEY AYGAAGSPP-K IPPNATLVFE VELFEFKGED LTEEEDGGII RRIRLGVKAM QGPNDGAMVE VALEGYHKDR LFDQRELCFE
p59       IAVATHKVGE LCRITCKPEY AYGSAGSPP-K IPPNATLVFE VELFEFKGED LTDDEDGGII RRIRTRGEGY ARPNDGAIVE VALEGYYKDR LFDQRELRFE
```

Figure 2 cont'd

hFKBP52	IGEGENLDLP YGLERAIQRM EKGEHSIVYL KPSYAFGSVG KEKFQIPPNA ELKYELHLKS FEKAKESWEM NSEEKLEQST IVKERGTVYF KEGKYKQALL291
bFKBP52	GEHSIVYL K YEIHLK GTVYF K
X17068	VGEGESLDLP CAWRRPFSAW RKESIPSCTS NLAMLLAVWG RRGSRSHRTA ELRYEVRLKS FEKAKESWEM SSARSWSRAT YVKERGTAYF KEGKYKQALL
X17069	VGEGESLDLP CAWRRPFSAW RKESIPSCTS NLAMLLAVWG RRGSRSHRTA ELRYEVRLKS FEKAKESWEM SSARSWSRAT YVKERGTAYF KEGKYKQALL
p59	VGEGESLDLP CGLEKAIQRM EKGEHSILYL KPSYAFGNAG KEKFQIPPYA ELKYEVHLKS FEKAKESWEM SSEEKLEQSA IVKERGTVYF KEGKYKQALI
hFKBP52	QYKKIVSWLE YESSFSNEEA QKAQALRLAS HLNLAMCHLK LQAFSAAIES CNKALELDSN NEKGLFRRGE AHLAVNDFEL ARADFQKVLQ LYPNNKAAKT391
bFKBP52	ALELDSN NEK
X17068	QYKKIVSWLE YESSFSGEEM QKVHALRLAS HLNLAMCHLK LQAFSAAIES CNKALELDSN NEKGLFRRGE AHLAVNDFDL ARADFQKVLQ LYPSNKAAKT
X17069	QYKKIVSWLE YESSFSGEEM QKVHALRLAS HLNLAMCHLK LQAFSAAIES CNKALELDSN NEKGLFRRGE AHLAVNDFDL ARADFQKVLQ LYPSNKAAKT
p59	QYKKIVSWLE YESSFSSEEV QKAQALRLAS HLNLAMCHLK LQAFSAAVES CNKALELDSN NEKGLFRRGE AHLAVNDFDL ARADFQKVLQ LYPSNKAAKA
hFKBP52	QLAVCQQRIR RQLAREKKLY ANHFERLAEE ENKAKAEASSG DHPTDTEHK EEQKSNTAGS QSQVETEA459
bFKBP52	LY ANHFERLAEE ETK ALVAAG DQPADAEH
X17068	QLAVCQQRTR RQLAREKKLY ANHFERLAEE EHKVKAEVAAG DHPTDAERK SLPRVWPPMD TKHQSLPTTH PHPHSSRPQ SHTSNQCNQC TCSHHCRSCS
X17069	QLAVCQQRTR RQLAREKKLY ANHFERLAEE EHKVKAEVAAG DHPTDAEHK GERN-NVAEN QSRVETEA
p59	QLAVCQQRIR KQIAREKKLY ANHFERLAEE ENKAKAEVAAG DHPMDTEMK DERN-DVAGS QSQVETEA
X17068	QAGHAGSSSS PSPGPPMKHP KPSVHSRHSP ARPSHRGSCPK NRKTFEGKV SKRKAVRRRK RTHRAKRRSS GRRYK

Figure 3

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-99 CCCGGCCTCCCGCACGCCCCGCAGGTAGCGCCCCCGCCCGCGGCCCAGAGTGCGCTCGCGCCGGCACCAGCTCCCGGATAAACGGCGCGCCCGCGCGGAGATGACAGCCGAGGAG

  16 ATGAAGGCGACCGAGAGCGGGGCGCAGTCGGCGCCGCTGCCCATGGAGGGAGTGGACATCAGCCCCAAACAGGACGAAGGCGTGCTGAAGGTCATCAAGAGAGAGGGCACAGGT
                                                                                                         1MetThrAlaGluGlu
   6 MetLysAlaThrGluSerGlyAlaGlnSerAlaProLeuProMetGluGlyValAspIleSerProLysGlnAspGluGlyValLeuLysValIleLysArgGluGlyThrGly
 130 ACAGAGATGCCCATGATTGGGGACCGAGTCTTTGTCCACTACACTGGCTGGCTATTAGATGGCACAAAGTTTGACTCCAGTCTGGATCGCAAGGACAAATTCTCCTTTGACCTG
  44 ThrGluMetProMetIleGlyAspArgValPheValHisTyrThrGlyTrpLeuLeuAspGlyThrLysPheAspSerSerLeuAspArgLysAspLysPheSerPheAspLeu
 244 GGAAAAGGGGAGGTCATCAAGGCTTGGGACATTGCCATAGCCACCATGAAGGTGGGGGAGGTGTGCCACATCACCTGCAAACCAGAATATGCCTACGGTTCAGCAGGCAGTCCT
  82 GlyLysGlyGluValIleLysAlaTrpAspIleAlaIleAlaThrMetLysValGlyGluValCysHisIleThrCysLysProGluTyrAlaTyrGlySerAlaGlySerPro
 358 CCAAAGATTCCCCCCAATGCCACGCTTGTATTTGAGGTGGAGTTGTTTGAGTTTAAGGGAGAAGATCTGACGGAAGAGGAAGATGGCGGAATCATTCGCAGAATACAGACTCGC
 120 ProLysIleProProAsnAlaThrLeuValPheGluValGluLeuPheGluPheLysGlyGluAspLeuThrGluGluGluAspGlyGlyIleIleArgArgIleGlnThrArg
 472 GGTGAAGGCTATGCTAAGCCCAATGAGGGTGCTATCGTGGAGGTTGCACTGGAAGGGTACTACAAGGACAAGCTCTTTGACCAGCGGGAGCTCCGCTTTGAGATTGGCGAGGGG
 158 GlyGluGlyTyrAlaLysProAsnGluGlyAlaIleValGluValAlaLeuGluGlyTyrTyrLysAspLysLeuPheAspGlnArgGluLeuArgPheGluIleGlyGluGly
 586 GAGAACCTGGATCTGCCTTATGGTCTGGAGAGGGCCATTCAGCGCATGGAGAAAGGAGAACATTCCATCGTGTACCTCAAGCCCAGCTATGCTTTTGGCAGTGTTGGGAAGGAA
 196 GluAsnLeuAspLeuProTyrGlyLeuGluArgAlaIleGlnArgMetGluLysGlyGluHisSerIleValTyrLeuLysProSerTyrAlaPheGlySerValGlyLysGlu
 700 AAGTTCCAAATCCCACCAAATGCTGAGCTGAAATATGAATTACACCTCAAGAGTTTTGAAAAGGCCAAGGAGTCTTGGGAGATGAATTCAGAAGAGAAGCTGGAACAGAGCACC
 234 LysPheGlnIleProProAsnAlaGluLeuLysTyrGluLeuHisLeuLysSerPheGluLysAlaLysGluSerTrpGluMetAsnSerGluGluLysLeuGluGlnSerThr
 814 ATAGTGAAAGAGCGGGGCACTGTGTACTTCAAGGAAGGTAAATACAAGCAAGCTTTACTACAGTATAAGAAGATCGTGTCTTGGCTGGAATATGAGTCTAGTTTTTCCAATGAG
 272 IleValLysGluArgGlyThrValTyrPheLysGluGlyLysTyrLysGlnAlaLeuLeuGlnTyrLysLysIleValSerTrpLeuGluTyrGluSerSerPheSerAsnGlu
 928 GAAGCACAGAAAGCACAGGCCCTTCGACTGGCCTCTCACCTCAACCTGGCCATGTGTCATCTGAAACTACAGGCCTTCTCTGCTGCCATTGAAAGCTGTAACAAGGCCCTAGAA
 310 GluAlaGlnLysAlaGlnAlaLeuArgLeuAlaSerHisLeuAsnLeuAlaMetCysHisLeuLysLeuGlnAlaPheSerAlaAlaIleGluSerCysAsnLysAlaLeuGlu
1042 CTGGACAGCAACAACGAGAAGGGCCTCTTCCGCCGGGGAGAGGCCCACCTGGCCGTGAATGACTTTGAACTGGCACGGGCTGATTTCCAGAAGGTCCTGCAGCTCTACCCCAAC
 348 LeuAspSerAsnAsnGluLysGlyLeuPheArgArgGlyGluAlaHisLeuAlaValAsnAspPheGluLeuAlaArgAlaAspPheGlnLysValLeuGlnLeuTyrProAsn
1156 AACAAAGCCGCCAAGACCCAGCTGGCTGTGTGCCAGCAGCGGATCCGAAGGCAGCTTGCCCGGGAGAAGAAGCTCTATGCCAATATGTTTGAGAGGCTGGCTGAGGAGGAGAAC
 386 AsnLysAlaAlaLysThrGlnLeuAlaValCysGlnGlnArgIleArgArgGlnLeuAlaArgGluLysLysLeuTyrAlaAsnMetPheGluArgLeuAlaGluGluGluAsn
1270 AAGGCCAAGGCAGAGGCTTCCTCAGGAGACCATCCCACTGACACAGAGATGAAGGAGGAGCAGAAGAGCAACACGGCAGGGAGCCAGTCTCAGGTGGAGACAGAAGCATAG
 424 LysAlaLysAlaGluAlaSerSerGlyAspHisProThrAspThrGluMetLysGluGluGlnLysSerAsnThrAlaGlySerGlnSerGlnValGluThrGluAla***
1381 CCCCTCTCCACCAGCCCTACTCCTGCGGCTGCCTGCCCCCCAGTCTCCCCACTCCACCCTGTTAGTTTGTAAAAACTGAAGAATTTTGAGTGAATTAGACCTTTATTTTTCTA
 495 TCTGGTTGGATGGTGGCTTTAGGGGAAGGGGGAAAGGTGTAGGCTGGGGGATTGAGGTGGGGAATCATTTTAGCTGGTGTCAGCCCCTCTTCCCTTCCTCCATTGCACATGAAC
 609 ATATGTCCATCCATATATATTCATCAGAATGTTAATTTATTTGCTCCCTCTGTTAGGTCCATTTTCTAAGGGTAGAAGAGGCAAGTGGTAGGGATGAGGTCTGATAAGAACCC
 723 AGGGTGGAGAGGGAGACTCCTGGGCAGCCGTTTTCCTCATCCTTTCCCTCTCCCAGTCCATTTCCAAATGTGGCCTCCATGTGGGTGCTAGGGACATGGGAAAAACCACTGCTA
 837 TGCCATTTCTTCTCTCTGTTCCCTTCCTCACCCCCGACGGTGTGGCTGATGATGTCTTCTGGTGTCATGGTGACCACCCCCTGTTCCCTGTTCTGGTATTTCCCCTGTCAGTTT
 951 CCCCTCTCGGCCAGGTTGTGTCCCAAAATCCCCTCAGCCTCTTCTCTGCACGTTGCTGAAGGTCCAGGCTTGCCTCAAGTTCCATGCTTGAGCAATAAAGTGGAAACA11
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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/08667

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C12N9/90; C07K15/08; G01N33/68
A61K37/02; A61K39/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	TRANSPLANTATION PROCEEDINGS vol. 23, no. 6, December 1991, pages 2890 - 2893 S.L. ROSBOROUGH ET AL. 'Identification of FKBP-related proteins with antibodies of predetermined specificity and isolation by FK 506 affinity chromatography' see page 2890, left column, paragraph 2 - right column, paragraph 1 see page 2891, right column, paragraph 2 - page 2892, right column, paragraph 1; figure 2	1,4
O,X	& PROCEEDINGS ON THE FIRST INTERNATIONAL CONGRESS ON FK-506 August 1991, PITTSBURGH, PA, USA	1,4

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 JANUARY 1993	
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9208667 SA 65987

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 19/01/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0379342	25-07-90	CA-A- 2008149	19-07-90
		JP-A- 2273181	07-11-90
		US-A- 5109112	28-04-92

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 379 342 (MERCK & CO. INC.) 25 July 1990 see page 2, line 43 - page 3, line 19 see page 9, line 24 - line 46	1-3,12, 19,21
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 15, 1 August 1991, WASHINGTON US pages 6677 - 6681 YONG-JIU JIN ET AL. 'Molecular cloning of a membrane-associated human FK506- and rapamycin-binding protein, FKBP-13' cited in the application see page 6677, left column, paragraph 2 - right column, paragraph 1 see page 6678, left column, last paragraph - right column, paragraph 2 see page 6680, right column, paragraph 2 -paragraph 4	1,2,4-6, 12,22,23